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MS APPEAL BRIEF - PATENTS
PATENT
4249-0103P

IN THE U.S. PATENT AND TRADEMARK OFFICE

In re application of Before the Board of Appeals
Ching M. CHUNG et al. Appeal No.:

Appl. No.: 09/788,476 Group: 1642
Filed: February 21, 2001 Examiner: Misook YU
Conf.: 6205
For: NOVEL GENES AND EXPRESSION PRODUCTS
 THEREFROM

REPLY BRIEF TRANSMITTAL FORM

MS APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

May 24, 2004

Sir:

Transmitted herewith is a Reply Brief (in triplicate) on behalf of the appellants in connection with the above-identified application.

☐ The enclosed document is being transmitted via the Certificate of Mailing provisions of 37 C.F.R. § 1.8.

The Examiner's Answer was mailed on March 24, 2003.

☒ Applicant claims small entity status. See 37 C.F.R. § 1.27.

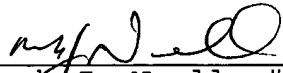
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Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

By 
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Attachment(s)

(Rev. 02/12/2004)



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REPLY BRIEF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

May 24, 2004

Sir:

This is a Reply to the Examiner's Answer that was mailed on March 24, 2004. The present invention is based upon identifying the correlation between the structure expressed (hcc-1 mRNA) and the functional state of the cell (cancerous). Thus, the present invention constitutes a valuable new diagnostic tool.

Although Applicants have discovered an important function of the hcc-1 gene – SEQ ID NO:1 – Applicants are not claiming the natural gene *per se*. However, Applicants are claiming the isolated gene in claim 1. Claim 1 also extends to certain variants of the natural gene. As disclosed e.g. in paragraphs [0047]-[0049] of the specification, the claimed variants can be used e.g. to target expression of the natural gene, in the course of treating hepatocellular

carcinoma and pancreatic adenocarcinoma. One such claimed variant is disclosed herein as having SEQ ID NO:3. Claim 1 extends to that and to similar variants that satisfy the structural (hybridization, % identity) and functional (expression) requirements of claim 1. In view not only of Applicants' important discovery but also of Applicants' extensive disclosure, Applicants have clearly provided the *quid pro quo* required to oblige the allowance of Claim 1 herein.

This Reply Brief will point out some of the major errors in the Examiner's Answer.

On page 4, the Examiner argues that "the only factor present in the claim is a partial structure in the form of a recitation of percent identity and hybridization". This argument completely ignores the claim's express functional requirement that "an mRNA corresponding to said nucleic acid is differentially or preferentially expressed in human hepatocellular carcinoma tissue or tissue from pancreatic adenocarcinoma relative to other tissue in said subject and/or in subjects not diagnosed with this condition".

In the paragraph bridging pages 5-6, the Examiner alleges that "it involves two steps to make the full scope of the claimed genus is: [sic] (1) to isolate other species by screening a large quantity of clinical samples ...; (2) to determine whether if [sic] the screened species meet the structural limitations of the instant claim". While the approach recognized by the Examiner would work, those skilled in the art could alternatively conduct the screening by determining first whether an "accused" nucleic acid was covered by claim 1 by determining whether the accused nucleic acid hybridizes to the reference sequences under the recited stringent hybridization conditions – a routine test. If the accused nucleic acid meets that exclusionary test, one skilled in the art would then verify by conventional means whether or not the accused nucleic

acid also meets the 60% sequence similarity requirement. An accused nucleic acid that passes both of these tests would then be subjected to a simple two sample test – that is, assayed for differential expression in normal tissue versus in carcinoma tissue. In connection with screening, it is noted that the amount of screening is not determinative. In the *Wands* decision, cited by the Examiner, the court indicated that enablement is not precluded by the necessity for experimentation, such as routine screening. The court confirmed that the experimentation needed to practice the invention must not be undue experimentation, but the court quoted the earlier decision in *In re Angstadt* (190 USPQ at 219), which had made the point that:

The key word is “undue”, not “experimentation”.

In re Wands, 8 USPQ2d at 1404.

On page 7, the Examiner argues that “The specification does not disclose the function of the claimed genus”. On the contrary, as disclosed for instance in paragraphs [0047]-[0049] of the specification, the claimed variants can be used for diagnostic purposes, and also for attenuating expression of the hcc-1 (e.g., by virtue of their antisense properties), in the course of treating hepatocellular carcinoma and pancreatic adenocarcinoma. The function of the claimed genus is thus either measurement of transcription of an hcc-1 gene of a subject or attenuation of expression of an hcc-1 gene of a subject.

On pages 7-9 of the Answer, the Examiner attempts to distinguish the present situation from that in Example 9 of the USPTO Written Description Guidelines. The Examiner points out that the claim in the Guidelines specifies a nucleic acid that encodes a protein, which protein has a specified effect. In Applicants’ claim 1, the nucleic acid also encodes a protein and expresses that protein. It is true that Applicants’ claim 1 does not indicate what effect the encoded, expressed protein has. However, Applicants’ claim 1 requires that mRNA encoding the expressed protein be expressed in amounts that differ in a

hepatocarcinoma or adenocarcinoma cell or tissue from the amounts that said mRNA is expressed in a normal cell or tissue. The Examiner's conclusion, that "There is no correlation between the chemical structure of the claimed genus and the recited function", is baseless. The structural similarity dictated by Applicants' claim 1 is correlated with the differential expression function recited therein. More specifically, the present invention claims diagnostic reagents. The structure of the claimed nucleic acids allows those skilled in the art to determine useful information about the state of the host.

On page 9 of the Answer, the Examiner alleges that "the instant claim excludes a non-naturally occurring nucleotide". It is respectfully submitted that the Examiner's position is based upon a misinterpretation of the claim. The host provides material to which the claimed invention is applied to perform diagnostics or to effect treatments. The claimed nucleic acid may incorporate a modified nucleotide, e.g. a biotinylated nucleotide for labeling and detection purposes.

Thus, in order to prevent a potential infringer from making use of Applicants' invention by substituting a nucleotide or nucleotide analog for one of the nucleotides stated in SEQ ID NO:1 or SEQ ID NO:3 or otherwise constructing a non-naturally occurring polynucleotide, Applicants are entitled to cover such conventional variants. Applicants have proposed claim 1 as a means to do so. If the Examiner can suggest another way in which Applicants can claim their invention in a manner to catch such manifest infringements, the Examiner is respectfully requested to suggest the alternative generic claim language.

Finally, on pages 10-13 of the Examiner's Answer, the Examiner appears to mix two concepts. The Examiner returns to the alleged screening of large quantities of clinical samples, which issue is addressed above at the bottom of

page 2 of this Reply Brief. Screening large numbers of samples, and even low success rates with such screens, does not establish undue experimentation. In *Wands*, the Board of Appeals had affirmed an Examiner's rejection, noting that only 2.8 percent of tested hybridomas were proved to fall within the claims. The Court of Appeals for the Federal Circuit indicated that the experimental technique used for testing the hybridomas was well known to those skilled in the art and that the experimentation involved was thus not undue. *In re Wands*, 8 USPQ2d at 1405-1406.

In the present situation, moreover, the predictability that the protein HCC-1 – or a naturally occurring allelic variant thereof (having a degree of identity sufficient to enable it to hybridize to the probe) – will be differentially expressed is high! One example showing the state of the art with respect to differential RNA expression screening assay technology may be found in Nuell et al., "Approach to the Isolation of Antiproliferative Genes", *Experimental Gerontology*, Vol. 24, pp. 469-476 (1989), a copy of which is enclosed for the Examiner's convenience. This paper demonstrates how to identify an isolated nucleic acid that is differentially expressed in two different tissues. See Figure 1. The Examiner here also questions "how to isolate an isolated nucleic acid" (Answer, page 12, bottom). It is respectfully submitted that the purification of nucleic acids is technology which is so well developed in the relevant art that no specific disclosure with respect thereto is necessary in the present situation.

For reasons set forth in the Brief on Appeal that was filed on 10 December 2003, the rejections of record are not sustainable.

Request for Oral Hearing

Applicants hereby respectfully request an oral hearing before the Board of Appeals for presenting arguments in connection with the present appeal in

the above-identified application.

A check in the amount of \$145.00 is enclosed for the filing fee in connection with the request for an Oral Hearing before the Board of Appeals.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

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Encl.: Nuell et al., *Exp. Ger.*, 24:469-476.

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APPROACH TO THE ISOLATION OF ANTIPROLIFERATIVE GENES

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Abstract — Poly(A) RNAs from normal rat liver and senescent human fibroblasts appear to have more antiproliferative activity than RNAs from regenerating rat liver and early passage human fibroblasts. We have screened two rat liver and one human liver library by differential hybridization and isolated four candidate cDNAs for this antiproliferative activity; one is fibronectin and three others do not match to any sequence in the mammalian portion of the GENBANK database. We are currently testing the antiproliferative nature of these cDNAs by microinjection of hybrid-selected RNA, and we describe an alternative strategy for cloning such genes based on construction of a cDNA library in an RNA expression vector.

Key Words: antiproliferative genes, RNA, fibronectin

INTRODUCTION

SEVERAL EXPERIMENTAL systems have provided evidence for genes whose absence results in increased cell proliferation and cancer, the tumor suppressor genes (Klein, 1987). A good example, and the most completely characterized of these loci to date, is the retinoblastoma (Rb) gene. Absence of both the maternal and paternal copy of the Rb gene is associated with childhood development of tumors of the eye (Lee *et al.*, 1987). Other tumor types have also been linked to the loss of both copies of a specific chromosomal region (Koufos *et al.*, 1984; Solomon *et al.*, 1987).

More direct experimental evidence for the presence of antiproliferative gene products has come from two other types of experiments: cell fusions and the microinjection of poly(A)-enriched RNAs. Several investigators have shown that when senescent and early passage cells are fused, both nuclei in each resulting heterokaryon have low tritiated thymidine uptake, suggesting that the nonproliferative phenotype of senescence is dominant (Norwood and Smith, 1985). Similarly, when poly(A)-enriched RNA fractions from senescent (Lumpkin *et al.*, 1986) or quiescent (Lumpkin *et al.*, 1986; Pepperkok *et al.*, 1988a) cells are injected into early

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passage (Lumpkin *et al.*, 1986) or HeLa cells (Pepperkok *et al.*, 1988a; Pepperkok *et al.*, 1988b), an inhibition of tritiated thymidine uptake is also observed in the nuclei of the injected cells. Normal rat liver cells also have an antiproliferative poly(A) RNA that is absent or reduced in cells from regenerating liver (Pepperkok *et al.*, 1988b; Lumpkin *et al.*, 1984).

In the present report, we describe the approach that we are taking to isolate cDNA clones of genes encoding antiproliferative mRNAs. This strategy combines differential colony hybridization, hybrid selection of specific mRNAs by cloned cDNAs, and a microinjection assay. An alternative strategy, involving the construction of a cDNA library in a vector for *in vitro* mRNA synthesis, is also described.

MATERIALS AND METHODS

Isolation of poly(A) RNA

Liver excised from 2-month-old male Sprague-Dawley rats was flash frozen in a dry-ice/butanol bath and broken into small pieces in a mortar filled with liquid nitrogen. The chunks of frozen tissue were homogenized using a polytron (Brinkmann) in 5 M guanidine isothiocyanate buffered to pH 6 with 25 mM sodium citrate and made 1% in beta-mercaptoethanol. Sarkosine was added to 0.5% after homogenizing. The homogenate was cleared by centrifugation at $16\,000 \times g$ and then total RNA was pelleted through an 8.5 ml cushion of 5.7 M CsCl (24 000 rpm in a Beckman SW28 for 40 h at 23 °). The pellet was redissolved in 10 mM Tris HCl (pH 7.5), 5 mM EDTA, and 1% lithium dodecyl sulfate, and extracted with an equal volume of chloroform:butanol 4:1. The emulsion was centrifuged at $16\,000 \times g$ for 15 min to separate the phases and the extraction was repeated until the interface was clear. The RNA was precipitated by the addition of 1/10 volume of 3M sodium acetate pH 5.5 and 2.2 volumes of ethanol, then stored in suspension at -80°C until further purification.

The preparation was enriched for polyadenylated RNA by a single passage over an oligo-dT column as described (Maniatis *et al.*, 1982), except that lithium salts were substituted for sodium in the buffers and elution was done with 0.05% lithium dodecyl sulfate (LDS) rather than with distilled water. Lithium salts were used because of the superior solubility of LDS compared to the sodium salt (SDS). The LDS was included in the elution solution to protect against nuclease degradation. Poly(A)-enriched RNA prepared in this manner typically results in a 40% reduction in the number of nuclei labeled by tritiated thymidine when microinjected (40 femtograms/cell) into the cytoplasm or early passage human fibroblasts (Lumpkin *et al.*, 1984).

To obtain polyadenylated RNA from regenerating liver, the same purification scheme was used, but the starting tissue was obtained 24 h after surgical removal of the two major lobes of the liver. Such RNA typically has a stimulatory effect upon the proliferation of fibroblasts (Lumpkin *et al.*, 1984).

Libraries

Three libraries were screened for potential antiproliferative cDNAs in these experiments. The first was constructed utilizing the technique of Buell (1978) and Land (1981). Starting material for this library was a poly(A)-enriched RNA fraction from normal rat liver that was further

enriched for polyadenylated RNA by a single passage over an oligo-dT column as described (Maniatis *et al.*, 1982), except that lithium salts were substituted for sodium in the buffers and elution was done with 0.05% lithium dodecyl sulfate (LDS) rather than with distilled water. Lithium salts were used because of the superior solubility of LDS compared to the sodium salt (SDS). The LDS was included in the elution solution to protect against nuclease degradation. Poly(A)-enriched RNA prepared in this manner typically results in a 40% reduction in the number of nuclei labeled by tritiated thymidine when microinjected (40 femtograms/cell) into the cytoplasm or early passage human fibroblasts (Lumpkin *et al.*, 1984).

Preparation of cDNA libraries

Poly(A)-enriched RNA was reverse transcribed using a hybridization probe. Specifically, the reaction mixture contained cold nucleotides, ^{32}P dCTP (Amersham Pharmacia Biotech), and contained 50 μl of 0.3 N NaOH. The reaction was neutralized with 0.3 N HCl. The cDNAs were precipitated with ethanol.

The labeled cDNA libraries were prepared by ligating each library into the filter-sterilized (or showing) purified for 1 h.

Computerized sequence analysis

Plasmids containing cDNA purified from human liver endonuclease unique *Eco*R sites were prepared by transfecting the polymerase chain reaction (PCR) for the sequence. The sequences were obtained from mammalian sequences (Pearson and Land, 1981) and also checked for accuracy.

Sequences were obtained from mammalian sequences (Pearson and Land, 1981) and also checked for accuracy.

Previous work on RNA from normal rat liver

al., 1988a; Pepperkok *et al.*, 1988b). The RNA that is absent or reduced in the nuclei of the injected cells is the same as that in the nuclei of the normal cells (Lumpkin *et al.*, 1984).

Using a microinjection assay, we were able to isolate cDNA clones of genes that are differentially expressed in normal and regenerating liver by using a differential colony hybridization and a microinjection assay. An 8.5 ml cushion of 5.7 M sucrose was cleared by centrifugation at 16 000 × g for 15 min to remove the interface. The RNA was extracted with an equal volume of 10 mM Tris HCl, pH 7.5, and 2.2 volumes of 100% ethanol.

The RNA was flash frozen in a dry-ice/liquid nitrogen. The chunks of RNA were then dissolved in 5 M guanidine isothiocyanate (GITC) in 1% beta-mercaptoethanol. The sample was cleared by centrifugation at 16 000 × g for 15 min to remove the interface. The RNA was extracted with an equal volume of 10 mM Tris HCl, pH 7.5, and 2.2 volumes of 100% ethanol.

The RNA was then subjected to a single passage over an oligo-dT column. The oligo-dT column was substituted for a dodecyl sulfate (LDS) rather than the superior solubility of LDS in the elution solution to protect the RNA. In this manner typically results in the loss of thymidine when microinjected into normal fibroblasts (Lumpkin *et al.*, 1984).

The same purification scheme was used for the removal of the two major lobes of the liver. The proliferation of fibroblasts

was enriched for antiproliferative activity by sucrose gradient size fractionation. The product cDNAs were ligated into the vector pUC8. The second library was a gift from Dr. Michael McPhaul and was constructed (McPhaul and Berg, 1987) from total mRNA from normal rat liver in the vector pCDV-1. The third was purchased from Clontech (Palo Alto, CA) and was made from total mRNA from human liver in the vector lambda gt11.

Preparation of radioactively labeled DNAs and library screening

Poly(A) RNA from normal and regenerating liver was made into probes for colony hybridization by synthesis of labeled cDNA with reverse transcriptase and ³²P-labelled dCTP. Specifically, 5 units of AMV reverse transcriptase (Pharmacia, Piscataway, NJ) was used in a reaction mixture of 25 µl containing 0.5 µg of poly(A) RNA (prepared as above), 0.5 mM of cold nucleotides (dATP, dGTP, and dTTP from Pharmacia, Piscataway, NJ), 50 µCi of alpha ³²P dCTP at 3000 Ci/mmol (New England Nuclear, Boston, MA), 0.5 µg oligo dT primer (Pharmacia), and 20 units of RNasin (Promega Biotech, Madison, WI). The reaction buffer contained 50 mM Tris HCl (pH 8.3 at 42 °C), 10 mM dithiothreitol, 10 mM MgCl₂, and 50 mM KCl. Reaction was for 1 h at 42 °C. The template RNA was then hydrolysed by addition of 175 µl of 0.3 N sodium hydroxide-2 mM EDTA and boiling for 3 min. The mixture was then neutralized with 50 µl of 10 M ammonium acetate, carrier tRNA (5 µg) was added, and the cDNAs recovered in TE buffer (10 mM Tris HCl, 1 mM Na₂EDTA, pH 7.5) following precipitation with 750 µl of 95% ethanol.

The labeled cDNA was used to screen replicate filters (Colony/Plaque Screen, DuPont) of cDNA libraries constructed from normal rat liver RNA. Approximately 20 000 clones from each library were screened in each experiment; hybridization and washing conditions were those of the filter supplier. Clones hybridizing to cDNAs from normal liver but failing to hybridize (or showing substantially reduced hybridization) to cDNAs from regenerating liver were purified for further characterization.

Computerized DNA sequence matching

Plasmids containing cDNAs of interest were grown in large amount in liquid culture and their DNA purified using standard techniques (Maniatis *et al.*, 1982). For the phage clones of the human liver library, the cDNA inserts were removed by digestion with the restriction endonuclease *Eco*RI and agarose gel purification, and the cDNA inserts were ligated into the unique *Eco*RI site of the vector pUC8. Clones from the rat liver libraries were sequenced directly from the original construct. Small amounts of each subcloned cDNA of interest were prepared by the alkaline lysis technique (Birboim and Doly, 1979) and partially sequenced using the Sanger (1977) approach. The enzyme used for sequencing was a cloned T7 DNA polymerase (Sequenase; United States Biochemical Corporation, Cleveland, OH). Conditions for the sequencing reactions were those of the supplier. Eighty to two hundred bases of sequence were obtained from each clone.

Sequences determined for the cDNAs of interest as above were each compared to the mammalian sequence collection of the GENBANK database using the FASTN program (Pearson and Lippman, 1988) on a VAX 780. The complementary sequence for each clone was also checked since the orientation of the DNA was unknown.

RESULTS

Previous work has shown that antiproliferative activity is present in the poly(A) fraction of RNA from normal rat liver and absent or substantially reduced in similar preparations from

cDNAs in these experiments. The results of Land (1981). Starting material from normal rat liver that was further

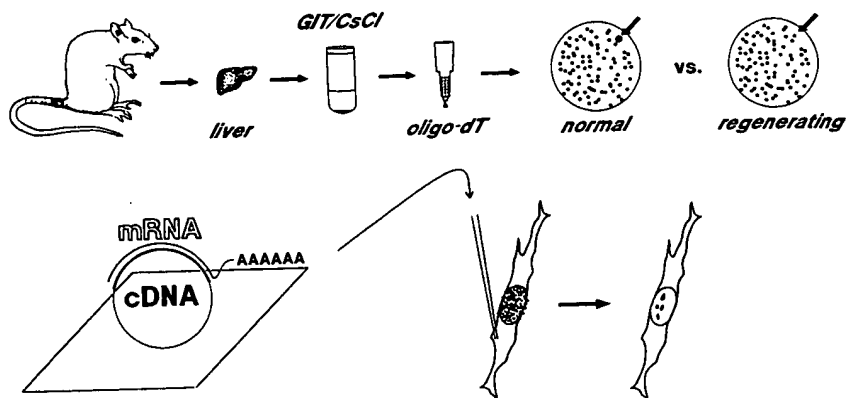
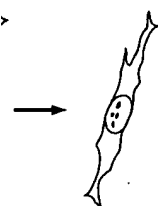
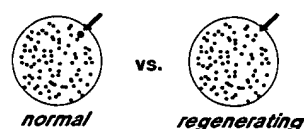


FIG. 1. Differential hybridization based strategy for molecular cloning of antiproliferative genes from rat liver. Poly(A) RNA is prepared from liver of either normal rats or rats partially hepatectomized 24 h earlier. Labeled cDNA is prepared from each pool of mRNA and is used to probe replica plated colonies of cDNA libraries expected to contain antiproliferative genes. Clones hybridizing to cDNA from normal but not regenerating liver tissue are purified and further screened by Northern analysis to confirm the initial result. DNAs from clones passing the screen can be fixed to a solid support and used as an affinity substrate to purify poly(A) sequences that are complementary in sequence to the cloned DNA. The hybrid selected mRNA can then be eluted and assayed for its ability to decrease tritiated thymidine incorporation into DNA after microinjection into cells in tissue culture.

regenerating liver (Pepperkok *et al.*, 1988b; Lumpkin *et al.*, 1984). This activity has been further characterized by size fractionation of poly(A) RNA on a sucrose gradient and has been found to sediment as if about the size of 18S ribosomal RNA (approximately 2 kilobases in length). In limiting dilution experiments, the abundance of the anti-proliferative message is estimated to be in the 0.01–0.1% range (Lumpkin *et al.*, 1984). Based upon these facts, we designed a cloning strategy using the technique of differential hybridization.

This strategy is presented schematically in Fig. 1. Three libraries were screened for potential antiproliferative cDNAs. The first library was made in the vector pUC8 using that fraction of sucrose gradient fractionated poly(A) RNA from normal rat liver that showed the most antiproliferative activity in microinjection experiments. The second library was made (by McPhaul) in the vector pCDV-1 using total poly(A) from normal rat liver. The third was made (by Clontech) in the vector lambda gt10 using total poly(A)-containing RNA from normal human liver. The rat liver libraries were screened using radioactive cDNA made using normal and regenerating rat liver mRNA as a template. The human liver library was screened using cDNA copies of RNA from senescent and proliferating human fibroblasts (IMR-90).

The results of these library screenings are shown in Table 1. It is clear that a number of false positive clones were initially selected. Several of the clones that passed two rounds of differential hybridization did not show a difference in expression in normal versus regenerating tissue when assayed in Northern hybridization experiments. In these Northern blots, a radioactive copy of the clone in question was hybridized to RNAs from either normal or regenerating liver that had been fractionated by size on a formaldehyde agarose gel and then transferred to a positively charged nylon membrane (Gene Screen Plus, DuPont). Other false positives gave no signal in Northern blots while a third group had insert cDNAs that were



cloning of antiproliferative genes from rats or rats partially hepatectomized (A) and is used to probe replica plated genes. Clones hybridizing to cDNA are further screened by Northern analysis and can be fixed to a solid support and re-complementary in sequence to the end assayed for its ability to decrease growth into cells in tissue culture.

TABLE 1. SCREENING FOR ANTIPROLIFERATIVE mRNAs^a

LIBRARY 1	LIBRARY 1	LIBRARY 2	LIBRARY 3
M4 ? ++	K6 fibrinogen	P1 ? -	C1 fibronectin
M5 ? ++	K13 ? -	P2 ? -	C2 fibronectin
M10 hybs to P5	K15 28S	P3 α2 MG	
M12 small	K17 Vit D BP	P4 ? -	
M15 cyt P450	K21 albumin	P5 cyt P450	
	K22 28S	P6 ? ++	
	K25 small	P7 albumin	
	K30 small	P8 albumin	
	K40 small	P9 ? +	

^aClones demonstrating greater hybridization to cDNA from normal liver RNA (or senescent IMR-90 RNA for library 3) than to cDNA from regenerating liver RNA (or early passage IMR-90 RNA for library 3) were subjected to DNA sequencing and were also used as probes in Northern blots. When the name of a gene is given, the DNA sequence of that clone was found to match (>85% identity over >100 bases) to the named gene in the GENBANK database. Other symbols and abbreviations are as follows: ?, no match (<70% identity) to GENBANK; 28S, match to 28S RNA; Vit D BP, vitamin D binding protein; α2 MG, alpha 2 macroglobulin; cyt P450, cytochrome P450; ++, 5 fold or greater differences in differential hybridization; +, 2 fold difference in hybridization; -, opposite pattern of hybridization to that desired; small, cDNA less than 40 bases in length; hybs to, hybridizes to.

et al., 1984). This activity has been

short to generate hybridization probes or unique DNA sequence.

To provide a means of prioritizing clones for hybrid selection, the DNA sequences of candidate cDNAs that showed the desired expression pattern (a higher level in normal than in regenerating liver RNA or a higher level in senescent than in early passage IMR-90 RNA) were determined and compared to the database of mammalian DNA sequences in GENBANK. The results of these DNA sequencing and database matching experiments are also presented in Table 1. Several of the clones turned out to be previously identified genes, such as albumin and cytochrome C, and both of the clones isolated from the human liver library were identified as fibronectin. Four of the clones remain unidentified.

The second library was made (by M. normal rat liver. The third was made poly(A)-containing RNA from normal human liver library was screened using human fibroblasts (IMR-90).

Figure 1. It is clear that a number of false clones that passed two rounds of expression in normal versus regenerating liver Northern blots, hybridized to RNAs from either normal or regenerating liver fibroblasts. Several clones were identified with the desired expression pattern and these were partially sequenced and compared by computer to the GENBANK database.

As shown in Table 1, cytochrome P450 and albumin were among the rat liver clones that were identified by our screening procedure. Because these genes have already been shown to have

DISCUSSION

Based on previous studies showing higher expression of antiproliferative mRNA in normal versus regenerating rat liver (Lumpkin *et al.*, 1984) and in senescent versus early passage human fibroblasts (Lumpkin *et al.*, 1986), a strategy was developed to clone potential antiproliferative cDNAs. Two rat liver libraries were screened by hybridization with radioactive cDNAs corresponding to mRNAs from either normal or regenerating liver; one human liver library was screened with cDNAs corresponding to mRNAs from senescent or early passage human fibroblasts. Several clones were identified with the desired expression pattern and these were partially sequenced and compared by computer to the GENBANK database.

As shown in Table 1, cytochrome P450 and albumin were among the rat liver clones that were identified by our screening procedure. Because these genes have already been shown to have

lower expression in regenerating liver than in normal liver (Panduro *et al.*, 1986), this is some reassurance that the screen is operating appropriately, that is, that it correctly identifies genes with the desired expression pattern (that may or may not be antiproliferative).

The identification of both of the clones from the human liver library as fibronectin is interesting. A similar result has been obtained independently from screening of a cDNA library made from RNA from senescent fibroblasts (J. Smith, unpublished data). It is possible that fibronectin is simply the most abundant mRNA that changes with *in vitro* aging, and that it has no antiproliferative activity, that is, analogous to the albumin and cytochrome clones found in the rat liver libraries. On the other hand, the addition of soluble fibronectin to endothelial cells in tissue culture has been shown to slow their growth (Madri *et al.*, 1988).

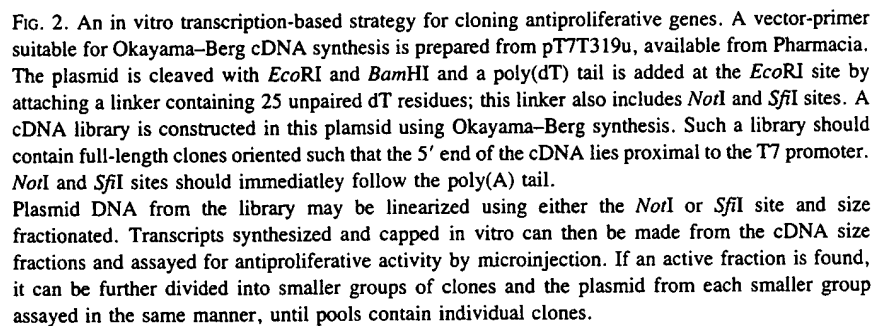
Three of the clones shown in Table 1 (working names M4, M5, and P6) have the desired pattern of expression and do not match any sequence in the GENBANK database. We are currently analyzing these clones by using them to purify their corresponding mRNAs (hybrid selection) for the microinjection assay.

It is possible that our screening procedure has failed to identify the antiproliferative cDNA(s) of interest. This might be because one or more of the assumptions underlying our cloning strategy is incorrect. Our principal assumption is that the difference in the antiproliferative activity of the RNA in, for example, normal versus regenerating liver is due to a difference in the amount of that RNA. However, it is possible that this difference is due to a change in the splicing pattern of the precursor RNA, as others in this issue have described the production of apparently inactive RNA with aging, for example, Seshadri and Campisi. If so, our cloning strategy might be ineffective, because both the active and inactive form of the message would share some sequence and thus could hybridize equally well to a cognate clone. A second assumption (this one based on some experimental evidence) is that the abundance of the antiproliferative mRNA is between 0.1 and 0.01% of the total mRNA in normal liver (McClung, unpublished). If the abundance is actually much lower, then we might have failed to identify the corresponding cDNA in our hybridization screening because of its very weak hybridization signal.

We are currently implementing an alternative cloning strategy that is less dependent on these assumptions. The essential elements of this approach are described in Fig. 2. In this scheme, cDNAs are cloned into a vector that can be used to generate a library of corresponding synthetic mRNAs, which can then be tested directly for antiproliferative activity by microinjection. Such an approach also avoids the need to prepare large amounts of DNA and poly(A) RNA, which is a drawback of hybrid selection-based strategies.

Specifically, cDNA is made by the Okayama-Berg technique (Okayama and Berg, 1982) using as template a sucrose gradient fraction of poly(A) RNA enriched for the antiproliferative activity. The Okayama-Berg approach optimizes the percentage of full-length clones in the library. Size-enriched RNA is used to increase the fraction of desired cDNAs, to compensate for the fact that cDNA cloning is not 100% efficient in generating clones that have the full protein-coding region. The cloning vector has a bacteriophage T7 promoter 5' to (upstream of) the cloning site as well as *NotI* and *SfiI* restriction enzyme sites at the 3' end of the gene (see Fig. 2). These rare cutting enzymes allow linearization of the plasmid to provide a terminus for RNA transcription *in vitro* while minimizing the risk of simultaneously cutting within the desired protein coding sequence. After construction of the library, plasmid from the whole library is linearized and size fractionated; this sizing step further enriches for the antiproliferative cDNA(s), because the antiproliferative activity bands at a single position in a sucrose gradient (Lumpkin *et al.*, 1984). RNA made *in vitro* from DNA of each size class is then

erg technique (Okayama and Berg, 1982). (A) RNA enriched for the antiproliferative he percentage of full-length clones in the action of desired cDNAs, to compensate for it in generating clones that have the full teriophage T7 promoter 5' to (upstream of, enzyme sites at the 3' end of the gene (section of the plasmid to provide a terminus for risk of simultaneously cutting within the on of the library, plasmid from the whole g step further enriches for the antiprolifer ity bands at a single position in a sucrose itro from DNA of each size class is there



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